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Differential chromosomal markers between sympatric karyomorphs of the fish *Hoplias malabaricus* (Bloch, 1794) (Characiformes: Erythrinidae)

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Abstract. Cytogenetic analyses were performed on *Hoplias malabaricus* (Bloch, 1794) from the Taquari River, Parana River drainage, revealing two sympatric karyomorphs. One karyomorph was characterized by 2n = 40 m/sm and 2n = 39 m/sm chromosomes for females and males, respectively, and an $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system. In the second karyomorph, specimens showed 2n = 42 m/sm chromosomes, without sex-related heteromorphism. Both karyomorphs were characterized by a distribution of heterochromatin in the pericentromeric and telomeric regions. In addition to the differences in the diploid numbers and the sex chromosomes, the GC-rich sites and the nucleolar organizer regions also showed clear differences between the karyomorphs. Coupled with the occurrence of unique chromosomal features within each karyomorph, the fact that hybrids have not been identified in the sampled population provides additional support of the existence of a species complex in *H. malabaricus*.

Key words: chromosomal banding, karyotype differentiation, species complex.

Introduction

Chromosomal rearrangements can play a major role in speciation processes, providing reproductive barriers and hence the formation of new biological species (Livingstone, Rieseberg, 2003). This fact has been observed in different biological groups, including house mice, where the accumulation of chromosomal rearrangements has been shown to lead to different "karyotypical races" or karyomorphs, and to a reproductive isolation between populations (Searle, 1998).

Amongst neotropical fish, several chromosomal changes have also occurred in the course of the karyotypical evolution of this group. In many cases, such rearrangements were responsible for the origin of different cytotypes (or different karyotypical forms), as observed in *Corydoras aeneus* (Gill, 1858) (Oliveira et al., 1988) and in other species. The sympatric occurrence of different cytotypes has been observed in neotropical fish as well, such as *Astyanax scabripinnis* (Jenys, 1842) (Maistro et al., 2000; Fernandes, Martins-Santos, 2006), *Astyanax* prope *fasciatus*

Cuvier, 1819 (Artoni et al., 2006), *Serrasalmus spilopleura* Kner, 1858 (Nakayama et al., 2000), *Laetacara* prope *dorsigera* (Heckel, 1840) (Martins-Santos et al., 2005), *Hoplerythrinus unitaeniatus* (Agassiz, 1829) (Giuliano-Caetano, Bertollo, 1988; Diniz, Bertollo, 2006), among others.

Among these fish, the species *Hoplias malabaricus* represents an interesting model for the study of chromosomal rearrangements. Many studies have been undertaken with the aim of analyzing the different cytogenetic events occurring in this species, that imply the existence of a species complex (Martins et al., 2006; Ferreira et al., 2007; Rosa et al., 2009a; Cioffi, Bertollo, 2010). Until now, seven karyomorphs have been reported, with thirteen cases of sympatric karyomorphs without the occurrence of hybrids (Bertollo et al., 2000). Among these karyomorphs, many are derived from chromosomal rearrangements, mainly centric fusions (Bertollo et al., 1997a). Also different systems of sex chromosome have been observed between the different karyomorphs, resulting from centric fusions and fissions (Bertollo et al., 2000; Rosa et al., 2009b; Cioffi et al., 2009a, b, 2010; Cioffi, Bertollo, 2010).

In order to provide a more detailed characterization of the diversity and to better understand the karyotypic evolution within this group, in the present work we investigated chromosomal markers in two karyomorphs of this species, collected in both sympatry and syntopy in Taquari River, a tributary of the Tibagi River (Paraná, Brazil), comparing our data with previous work.

Material and methods

Forty-seven specimens of *Hoplias malabaricus* were collected in the Taquari River, Tibagi River basin (23°10'45"S/50°56'30.9"W), Jataizinho, Paraná State, Brazil (Fig 1). The specimens analyzed are deposited in the museum of zoology at the Universidade Estadual de Londrina, under the number MZUEL 5142.

Conventional staining. Metaphase chromosomes were obtained by the air drying technique (Bertollo et al., 1978) and lymphocyte culture (Fenocchio, Bertollo, 1988) and stained with 5% Giemsa in phosphate buffer (pH 6.8) The chromosomes were organized as metacentric (m) and submetacentric (sm) for the preparation of a karyogram.

Chromosome banding. The distribution of heterochromatin was analyzed by Giemsa C-banding after treatments with 0.1 M HCl, $Ba(OH)_2$ and 2× SSC (Sumner, 1972). The silver staining were detected according to Howell and Black (1980). The GC- and AT-rich bands were detected with chromomycin A_3 (CMA_3) and 4'-6-diamino-2-phenylindole (DAPI), respectively, according to Schweizer (1980). The slides were stained with 0.5 mg/mL CMA_3 for 1.5 h, washed in distilled water and sequentially stained with 2 µg/mL DAPI for 30 min. Slides were mounted with a medium composed of glycerol/McIlvaine buffer (pH 7.0) 1:1, plus 2.5 mM $MgCl_2$.

Fluorescent in situ hybridization. The *in situ* hybridization procedure was performed according to Swarça et al. (2001). The 18S rDNA probe of *Prochilodus argenteus* Spix & Agassiz, 1829 (Hatanaka, Galetti Jr., 2004) was labeled with biotin-14-dATP by nick translation. Slides were treated with 30 µL of hybridization mixture containing 100 ng of labeled probe (4 µL), 50% formamide (15 µL), 50% polyethylene glycol (6 µL), 20× SSC (3 µL), 100 ng of calf thymus DNA (1 µL) and 10% SDS (1 µL). The material was denatured at 90 °C for 10 min, and hybridization was performed overnight at 37 °C in a humidified chamber. Post-hybridization washes were

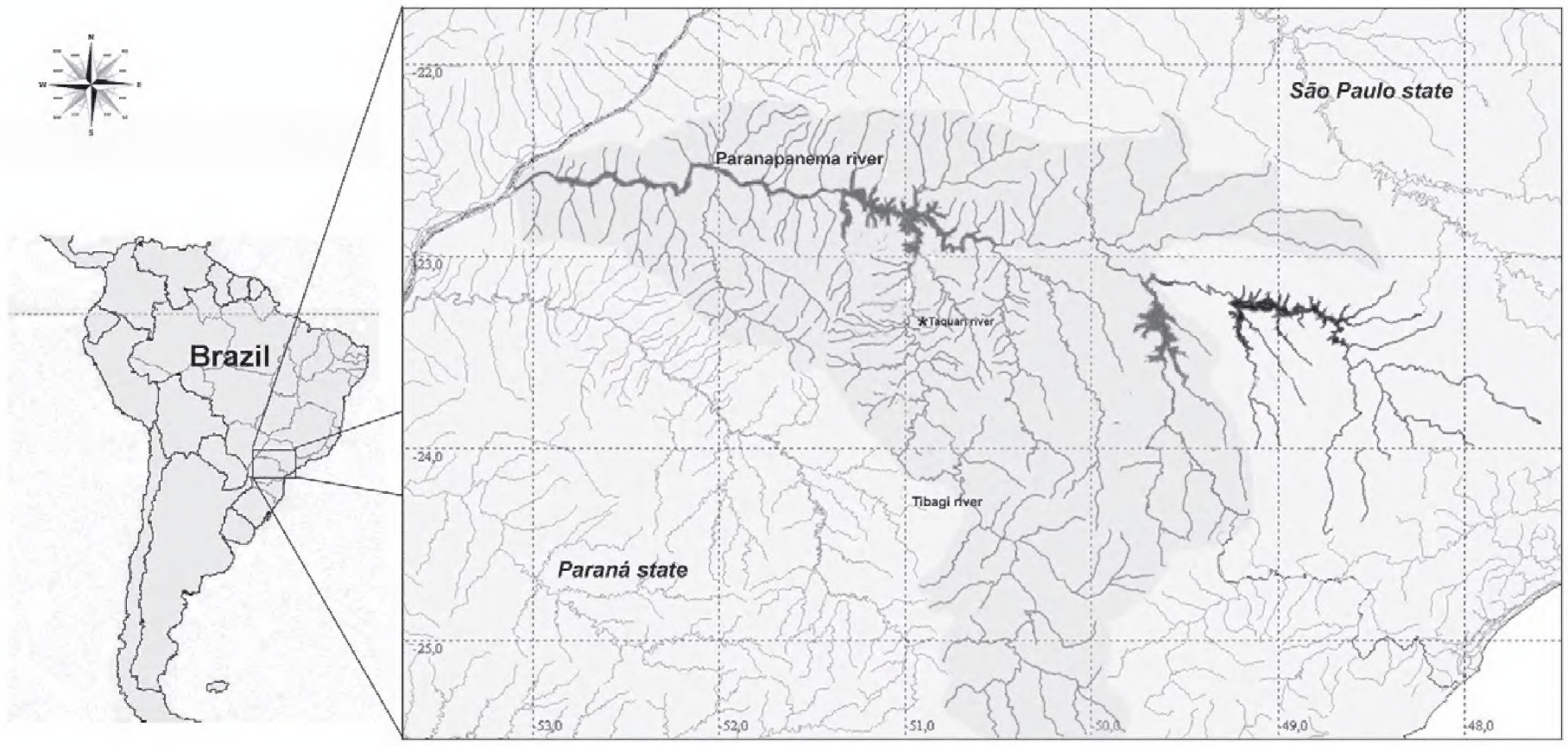

Fig. 1. Hydrographic map showing lower the Paranapanema river basin (shaded) and the collection site of *Hoplias malabaricus* (*) in the Taquari River.

carried out in 2× SSC, 20% formamide in 0.1× SSC, 0.1× SSC and 4× SSC/0.2% Tween 20, all at 42°C. The probe was detected with a solution of 5% BSA and FITC-conjugated avidin (50:0.5, v:v). The post-detection washes were performed in 4× SSC/0.2% Tween 20 at room temperature. Slides were mounted with 25 μL of a medium composed of 23 μL of DABCO solution (1,4-diaza- bicyclo (2.2.2)-octane (2,3%), 20 mM Tris HCl, pH 8.0, (2%) and glycerol (90%), in distilled water), 1 μL of 2 μg/mL DAPI and 1 μL of 50 mM $MgCl_2$.

All the images were acquired with a Leica DM 4500 B microscope equipped with a DFC 300FX camera and Leica IM50 4.0 software, and optimized for best constrast and brightness with iGrafx Image software.

Results

Two karyomorphs were observed among the examined specimens. In the first case, fourteen females presented a diploid number of 2n=40 m/sm chromosomes (Fig. 2, Aa), and twelve males had a diploid number of 2n=39 m/sm chromosomes, showing the occurrence of a $X_1X_1X_2X_2/X_1X_2Y$ multiple sex chromosome system (Fig. 2, Ba). The second karyotypical form comprised 2n=42 chromosomes (Fig 2, Ca), without chromosomal differentiation between sexes (10 males and 11 females). According to Bertollo et al. (2000), these forms would correspond to karyomorphs D and A, respectively.

Heterochromatin blocks were observed at the centromeric regions of all chromosomes and at the telomeric regions of some pairs. In the karyomorph D, the X_1 and X_2 chromosomal pairs, corresponding to pairs 6 and 20, respectively, presented centromeric blocks (Fig. 2, Ab), while the Y chromosome showed, besides a centromeric C-band, an interstitial heterochromatin segment on long arms (Fig 1, Bb). In specimens belonging to karyomorph A (2n=42), besides the centromeric heterochromatic segments, telomeric C-bands

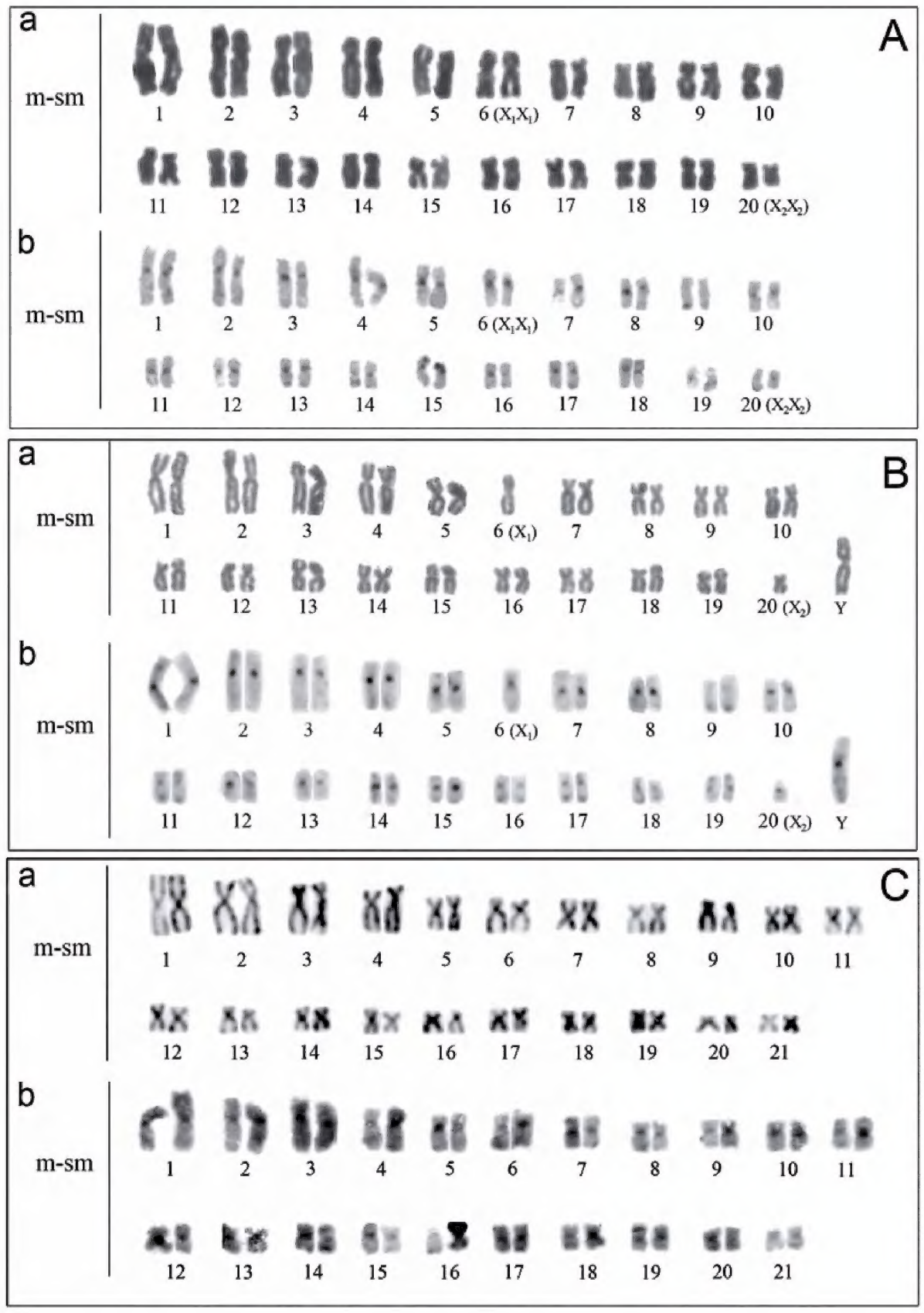

Fig. 2, a, b. Karyotype of *Hoplias malabaricus* from Taquari River after conventional staining (**a**) and C-banding (**b**). (A) female – cytotype D (2n = 40); (B) male – cytotype D (2n = 39); (C) male/female – cytotype A (2n = 42).

were observed in the 14th pair (Fig. 2, Cb).

Both karyomorphs were characterized by the presence of multiple Ag-NORs (Fig. 3, a-c). However, the occurrence of bitelomeric NORs, i.e., in both telomeres of the same chromosome, was identified only within karyomorph A (Fig. 3, c).

GC-rich blocks were evident in several chromosomes of karyomorph D, in both males and females. The X chromosomes presented a larger fluorescent signal when compared to the others chromosomal pairs (Fig. 3, d, e). This block was more evident in males than females, whereas the Y chromosome presented two GC-rich segments in an interstitial position, one on the long arm and the other on the short arm

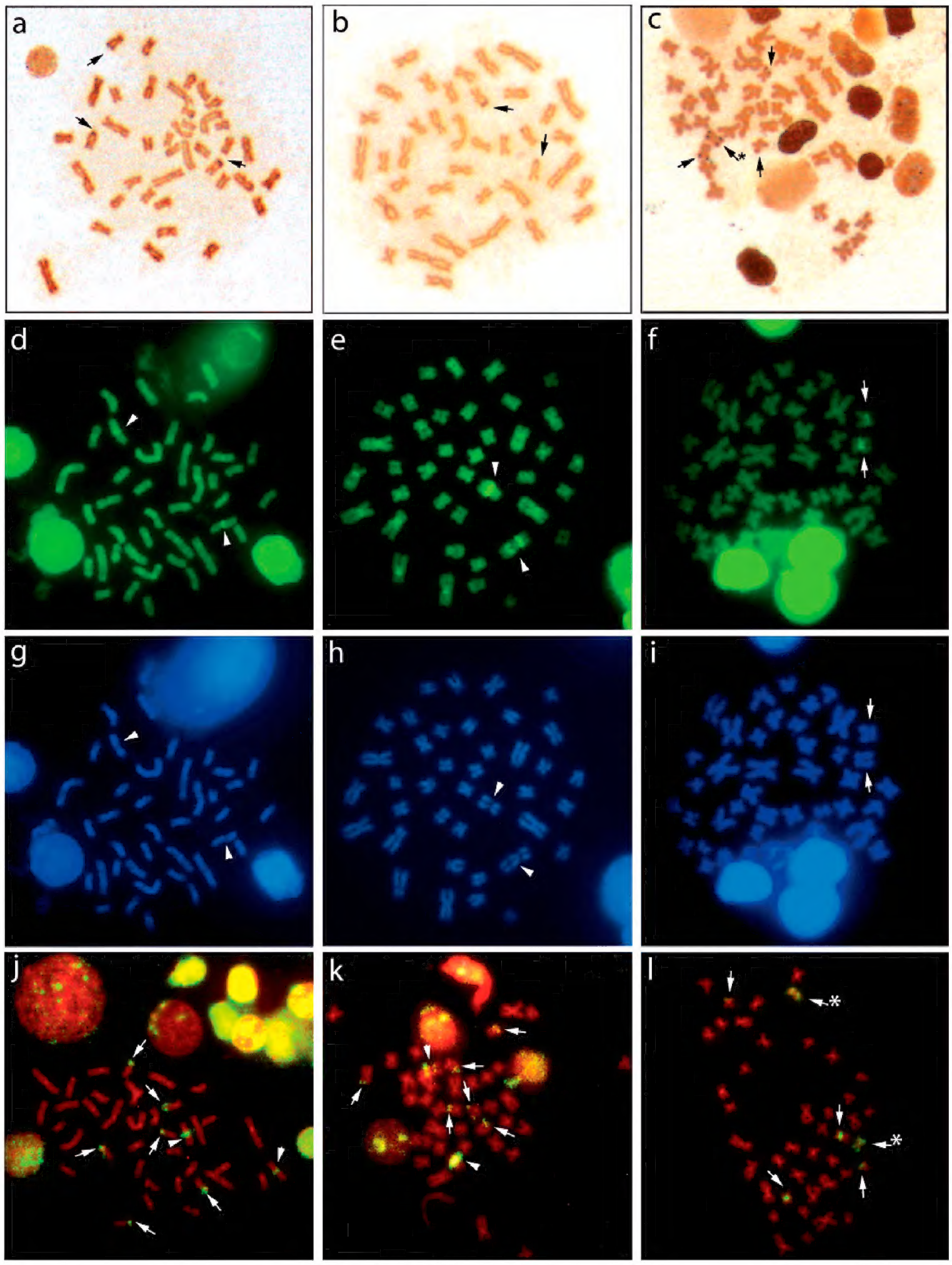

Fig. 3, a-l. Metaphases of *Hoplias malabaricus* from the Taquari River. **a**, **b**, **c** - Ag-NORs in females from cytotype D, males from cytotype D and males/females from cytotype A, respectively. **d**, **e**, **f** - CMA_3 staining in females from cytotype D, males from cytoype D and males/females from cytotype A, respectively. **g**, **h**, **i** - DAPI staining in females from cytotype D, males from cytoype D and males/females from cytotype A, respectively. **j**, **k**, **l** - FISH with 18S rDNA probe in females from cytotype D, males from cytotype D and males/females from cytotype A, respectively. The arrows indicate the NORs, the head arrows indicate the putative sex chromosomes and the asterisks indicate the bitelomeric NORs.

Table 1. Populations of *Hoplias malabaricus* bearing sympatric cytotypes. (*) according to Bertollo *et al.* (2000); (-) not identified

Locality	Sympatric cytotypes*	Chromosomal number	Sex chromosome system	References
Aguarapey River (Corrientes – Argentina)	A C	42 m/sm 40 m/sm	- -	Lopes et al., 1998
Igarapé Mindu (Manaus-AM)	A C	42 m/sm 40 m/sm	- -	Bertollo et al., 2000
Claro River (Tamarana-PR)	A C	42 m/sm 40 m/sm	- -	Rosa, 2006
Mogi-Guaçu River (Luiz Antonio-SP)	A D	42 m/sm 39-40 m/sm	- $X_1X_1X_2X_2/X_1X_2Y$	Scavone et al., 1994
Grande River (Conceição das Alagoas-MG)	A D	42 m/sm 39-40 m/sm	- $X_1X_1X_2X_2/X_1X_2Y$	Dergam, 1996
Taquari stream (Londrina-PR)	A D	42 m/sm 39-40 m/sm	- $X_1X_1X_2X_2/X_1X_2Y$	Present paper
Paraná River (Porto Rico-PR)	A C D	42 m/sm 40 m/sm 39-40 m/sm	- - $X_1X_1X_2X_2/X_1X_2Y$	Pazza, Júlio Jr, 2003
Aripuanã River (Aripuanã-MT)	C G	40 m/sm 40-41 m/sm	- XX/XY_1Y_2	Bertollo et al., 1997a Bertollo et al., 2000
Madeira River (Porto-Velho-RO)	C G	40 m/sm 40-41 m/sm	- XX/XY_1Y_2	Bertollo et al., 2000
São Francisco River (Três Marias-MG)	A F	42 m/sm 40 m/sm	- -	Dergam, Bertollo, 1990 Bertollo et al., 2000
Tocantins River (Tucuruí-PA)	C F	40 m/sm 40 m/sm	- -	Bertollo et al., 1997b
Trombetas River (Porto Trombetas-PA)	E G	42 m/sm 40-41 m/sm	- XX/XY_1Y_2	Bertollo et al., 2000
Lagoa Carioca (Parque Estadual do Rio Doce-MG)	B D	42 m/sm 39-40 m/sm	XX/XY $X_1X_1X_2X_2/X_1X_2Y$	Born, Bertollo, 2006

(Fig. 3, e). On the other hand, the karyomorph A presented a single GC-rich block, probably located on the sixth pair (Fig. 3, f). All GC-rich segments were negatively stained by DAPI, and AT-rich bands were absent in both karyomorphs (Fig. 3, g, h, i).

Eight 18S rDNA cistrons were evident in the karyomorph D, occupying the telomeric regions of three chromosomal pairs in both males and females. Additionally, in females, two interstitial sites on the long arm of a chromosomal pair were detected close to centromeres (Fig. 3, j). In males, this feature was observed in a single chromosome, while another chromosome presented interstitial sites on both arms (Fig 3, k). Probably, these chromosomes would correspond to X_1 and Y, respectively. In karyomorph A, six 18S rDNA-bearing chromosomes were detected, comprising two telomeric, two interstitial and two bitelomeric sites (Fig. 3, l).

Discussion

Several cytogenetic studies have been carried out in order to understand the

remarkable karyotype diversity in the species *Hoplias malabaricus* (Santos et al., 2009; Rosa et al., 2009a; Blanco et al., 2010a). Such chromosomal diversity is characterized by the occurrence of distinct sex chromosome systems and the identification of at least seven karyomorphs, classified from A to G (Bertollo et al., 2000). The samples of *Hoplias malabaricus* from the Taquari River presented two sympatric karyotypic forms, characterized as karyomorph A (2n=42) and karyomorph D (2n=39/40).

Other examples of sympatric karyomorphs have been reported within *H. malabaricus* (Table 1). Bertollo et al. (2000) listed nine occurrences throughout Brazil and Argentina. Recent studies have also reported new cases in the Upper Paraná basin (Pazza, Júlio Jr., 2003), in the Tibagi River basin (Rosa, 2006) and in the Parque Estadual do rio Doce (Born, Bertollo, 2006). No possible hybrids have been identified in any of these cases, indicating that such distinct karyotypic forms are likely to be reproductively isolated. Investigations using RAPD markers within a population of *H. malabaricus* presenting the same sympatric karyomorphs analyzed in the present study, demonstrated a lack of gene flow between karyomorphs, reinforcing their putative reproductive isolation (Dergam, 1996).

The general location pattern of pericentromeric and telomeric heterochromatin detected in specimens from the Taquari River is usually found in different populations of *H. malabaricus* (Lopez, Fenocchio, 1994; Born, Bertollo, 2001; Vicari et al., 2003, 2006), although minor differences may also occur among karyomorphs/populations.

However, the most contrasting features between both karyomorphs here analyzed refer to the nucleolar organizer regions and location of GC-rich chromosomal sequences. While both males and females from the karyomorph D presented 2-3 telomeric Ag-NORs on long arms of submetacentric chromosomes, specimens from karyomorph A were characterized by the additional occurrence of bitelomeric Ag-NORs. A bitelomeric Ag-NOR pattern was previously reported by Born and Bertollo (2006) in samples of the same karyomorph, found in sympatry with other karyotypical form (karyomorph B: 2n=42, XX-XY), in Parque Estadual do rio Doce.

Data based on FISH results also reinforce the differential location and number of NORs between the two karyomorphs. In fact, eight 18S rDNA-bearing chromosomes were detected in males and females from karyomorph D, and two of them probably correspond to the X_1 and Y chromosomes. According to Bertollo et al. (1997a), the formation of the Y chromosome in males of karyomorph D was related to a centric fusion between one X_1 and one X_2 chromosome. The Y chromosome in the karyomorph D from the Taquari River might have originated by a similar rearrangement. However, once the 18S rDNA sequences are located on both arms of the putative Y chromosome and ribosomal genes are absent in the X_2 chromosome, we hypothesize that a pericentric inversion might have also taken place, moving a portion of the 18S rRNA genes to the short arms of the Y chromosome.

On the other hand, six chromosomes carrying 18S rDNA sites were observed within karyomorph A, two of them in a bitelomeric location, in agreement with the pattern observed in two populations of the same karyomorph in the Tibagi (PR) and Ribeira (SP) rivers (Vicari et al., 2005). Blanco et al. (2010b) showed a differentiation in the number of ribosomal sites in three different populations of *H. malabaricus*, and differences in the distribution of 5S rDNA and 5S*Hind*III satellite DNA were also observed.

The GC-rich chromosomal regions also showed a distinct pattern of location between karyomorphs. Males and females of karyomorph D, besides several reduced blocks distributed throughout the karyotype, showed more conspicuous signals related to the putative sex chromosomes, apparently corresponding to C-bands and 18S rDNA sites. However, in karyomorph A, only a single chromosomal pair showed more evident GC-rich band, located at centromeric region.

Interpopulation variation has been reported in many groups of neotropical fish, and this feature has been commonly correlated with population structure (Galetti Jr. et al., 1994). Species with limited and sedentary populations are more likely to present a greater degree of variation when compared to those with large populations of high mobility. This feature could explain the karyotypical diversity found in *H. malabaricus*, since it represents a low-vagility species, inhabiting lentic environments. Consequently, the fixation of chromosomal rearrangements would be easily accomplished, leading to the remarkable karyotypical plasticity observed in different populations of *H. malabaricus* and favoring the occurrence of distinct karyotypical forms. In sympatric conditions, such differences could be sufficient to prevent the gene flow between different karyomorphs (McCoy, 2003). The fact of the two karyomorphs occur in sympatry and there are no intermediate karyotypic forms, indicates that there must be reproductive isolation, so they are probably different species.

Therefore, our data corroborate the current suggestion that *H. malabaricus* comprises a species complex of independent evolutionary units (Bertollo et al., 2000). The available information on karyotypic structure and chromosomal markers, along with further morphological and molecular studies, such as mtDNA analysis, are important to provide a more reliable phylogeny of this complex fish group.

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